ARTICLE

Responses of Fertile and Sterile Screwworm (Diptera: Calliphoridae) Flies to Bovine Blood Inoculated with Bacteria Originating from Screwworm-Infested Animal Wounds

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ABSTRACT A simple bioassay system was developed to study locomotory and ovipositional responses of screwworm, *Cochliomyia hominivorax* (Coquerel), flies to bovine blood inoculated with eight species of coliform bacteria that were isolated from screwworm-infested animal wounds. When exposed to odors from bacteria-inoculated blood which was incubated for 72 h at 37°C, ≈50% of 7- and 10-d-old gravid females landed on the blood by the end of 15 min test exposure. Only 17% of 7-d-old reproductively sterile females (from irradiated pupae) with previtellogenic ovaries and 2% of 4-d-old vitellogenic females responded to the same treatment. Females generally reacted in greatest numbers to bacteria-inoculated blood incubated for 72 h, followed by 48 h, then 24 and 96 h. Males of all ages tested were unresponsive. Although oviposition occurred in tests with gravid females lasting for 1 h, with both inoculated blood and an uninoculated control, the inoculated sample was significantly better than the control at 48, 72, and 96 h incubation duration. Our results are consistent with the conclusion that the inoculated blood, when incubated for 48–72 h, gives off volatile chemicals which attract gravid females and contains an oviposition stimulant that acts following contact and feeding. The volatiles, once isolated and identified, may be useful for sampling gravid females in the field as well as improving the oviposition system in the mass-production facility of the screwworm eradication program.

KEY WORDS *Cochliomyia hominivorax*, attractant, bacteria, oviposition stimulant

THE SCREWWORM, *Cochliomyia hominivorax* (Coquerel), is an economically important pest of cattle. Larvae feed as obligate parasites on living tissue of animals. Gravid female flies are especially attracted to screwworm-infested wounds and the navels of newborns where the flies oviposit (Bushland 1960). Infested wounds appear to release attractive odors that act as host-finding cues. Also, the wound's chemicals appear to play a role in stimulating oviposition (DeVaney et al. 1970, 1973; Eddy et al. 1975; Bromel et al. 1983; Hammack and Holt 1983; Hammack et al. 1987; Hammack 1991). Gravid females are also attracted by odors from decomposing meat and liver (Bishopp 1937) and spent artificial diet from larval rearing (Adams et al.1979). Screwworm flies oviposit in response to other odors such as ammonium carbonate (DeVaney et al. 1970), spent larval diet (Mackley and Snow 1982) and cultures from a variety of bacterial species (Eddy et al. 1975). Holt et al. (1979) and Hammack (1991) reported that fresh uninoculated blood stimulates oviposition on contact but is not olfactorily attractive to screwworm flies. Cultures of gram-negative bacteria associated with screwworms produce attractive odors and, therefore, bacterial contamination of oviposition substrates may be a prerequisite for screwworm activity. Fresh wounds, fresh liver, or sterile blood are not olfactorily attractive (DeVaney et al. 1973, Eddy et al. 1975, Bromel et al. 1983, Hammack and Holt 1983, Hammack et al. 1987). DeVaney et al. (1973) and Eddy et al. (1975) showed that the source of odors in incubated blood attractive to gravid screwworm flies was bacteria and/or compounds produced by them. These authors found *Providencia rettgeri* (Hadley, Elkins & Caldwell) cultures the most attractive of all the bacteria cultures tested. Bromel et al. (1983) isolated several species of bacteria from screwworm egg masses, larvae, pupae, and from fluids associated with screwworm-infested animal wounds and artificial rearing medium. Their olfactometer studies indicated that the culture medium in which *P. rettgeri* was grown was attractive to gravid female flies. Hammack (1991) reported that fresh bovine blood stimulated as many (or more) females to oviposit as did cultures of *P. rettgeri* or the fluid from screwworm-infested wounds, a favored oviposition site in nature. Preliminary tests in large rearing cages demonstrated that fresh bovine blood, inoculated with bacteria originating from screwworm-infested animal wounds, attracts and stimulates gravid screwworm females to oviposit in large quantities (J.B.W., unpublished data).

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In this article, we report the attractiveness to gravid screwworm flies of bovine blood, inoculated with eight species of bacteria that originated from screwworm-infested animal wounds. The purpose of this research was to develop a simple bioassay system to test the olfactory response of gravid screwworm flies to the bacteria-inoculated blood, to test the response of sexually sterile females, to determine the incubation time necessary for the inoculated blood to have maximum response, and to investigate the role of the bacteria-inoculated bovine blood as an oviposition stimulant. An ultimate goal is to improve the oviposition system for mass-rearing facility of the screwworm eradication program through isolating active chemical(s) from volatiles of bacterial cultures as well as identifying some contact oviposition stimuli from the inoculated blood. Chemicals from the volatiles once isolated and identified may also be useful for sampling gravid females in field studies.

Materials and Methods

Insects. All tests were conducted using flies of the Panama-95 strain supplied by the Methods Development Department of the mass-rearing facility in Tuxtla Gutierrez, Mexico. This strain was developed by the ARS from wild females collected in Panama in 1995 and transferred to the Methods Development Department in July 1996. Larvae were reared on a gelled diet prepared with spray-dried whole bovine blood, spray-dried chicken egg, and a milk substitute as described by Chaudhury and Alvarez (1999). Adults were maintained at 25 ± 1°C, 50 ± 5% RH, and a photoperiod of 12:12 (L:D) h, and were fed on a mixture of horse meat and honey (Chaudhury et al. 2000). Sterile flies of the Panama-95 strain were obtained from the pupae which were irradiated using a gamma irradiation source available at the mass-rearing plant. Flies were separated by sex on the day of testing without using anesthesia.

Bacteria. Bacteria isolated from screwworm infested animal wounds (J.B.W., unpublished data) belonged to the family Enterobacteriaceae, gram-negative coliform rods bacteria. The species were: *Enterobacter cloacae* Weigmann, *Enterobacter sakazakii* (Cohn), *Klebsiella oxytoca* (Flugge), *Proteus mirabilis* Hauser, *Proteus vulgaris* Hauser, *Providencia rettgeri* (Hadley, Elkins, & Caldwell), *Providencia stuartii* (Buttiaux, Osteux, Fresnoy, and Moriamez) and *Serratia liquefaciens* (Katznelson)

Blood. Fresh bovine blood was collected from a local slaughter house and treated with EDTA at the rate of 2 g/liter of blood. The blood was kept refrigerated at 5°C until used on the following day.

Preparation of Samples. Samples of each species of bacteria were grown on agar slants in culture tubes for 24 h at 37°C. Each slant culture was then transferred to 30 ml of nutrient broth (Bioxon de Mexico, Oaxaca) in a 100-ml Erlenmeyer flask, which was agitated for 24 h at 37°C using a mechanical agitator (Labline Instruments, Melrose Park, IL) set at 150 rpm. Two ml of bacterial broth from each flask was then pipetted into 84 ml of blood in a 150-ml Erlenmeyer flask to make a 100-ml mixed bacterial sample, which was incubated at 37°C for 24, 48, 72, or 96 h before use in bioassays. The control samples were prepared with 84 ml of blood and 16 ml of sterile broth and were incubated and handled the same way as the treated samples.

Bioassay for Flight Response. Bioassays were conducted in wire mesh cages (1 by 1 by 1 m) built with a solid metal base and a 20-cm-diameter opening on the front wall situated close to the base. The opening was covered and secured with a sleeve of stretch cotton/polyester material. Four cages were set up in a row with at least a 1-m space between the cages. The room was kept well lighted during the tests by overhead fluorescent lamps (500 lux at the roof of the cage). The temperature and humidity of the room were maintained at 26 ± 1°C and 55 ± 5% RH, respectively. The bioassays were conducted between 0900 and 1300 hours. Cages were washed with hot water and the sleeves were replaced after each test.

One hundred flies of known age, sex, and reproductive status (fertile or sterile) were introduced into each cage and were allowed to settle in the new environment for 5 min. Meanwhile, each of the incubated blood samples (control or bacteria-inoculated) was poured into a paper towel lined plastic tray (21 by 15 cm, 3 cm deep). The tray with the sample was then placed into a larger plastic tray (21.5 by 15.5 cm, 12 cm deep) with hot water (60°C) to keep the blood sample warm (39–41°C) during the bioassay. The control sample tray (blood without added bacteria) was placed on the bottom in the cage. Behavior of the flies was observed during a 15-min period immediately following placement of the sample in the cage. The number of flies remaining on the tray after 15 min was recorded as the number attracted. The tray was then removed from the cage, taking care that no flies were removed with it. After 10 min, a treated sample tray (blood with bacteria) was introduced into the same cage and the observation was repeated for another 15 min. Number of egg masses, if any, laid during the 15-min test period were also recorded. Six to eight tests were conducted per insect group (4-, 7-, and 10-d-old mated males and fertile females, and 7-d-old sterile females) and incubation period (24, 48, 72, and 96 h postinoculation) for the blood. Percentage response was calculated as follows:

$$\frac{\begin{pmatrix}\text{No. of flies}\\ \text{landing on}\\ \text{treated tray}\end{pmatrix} - \begin{pmatrix}\text{No. of flies}\\ \text{landing on}\\ \text{control}\end{pmatrix}}{(\text{No. of flies tested})} \times 100$$

Oviposition Test. These tests were conducted in smaller cages (0.4 by 0.4 by 0.7 m), built similarly as above, with 200 gravid females (7 d old) per test. In this case, both the test and control sample trays were introduced simultaneously into the cage where females were given the opportunity to oviposit for 1 h. The sample trays were slightly modified by including a small piece of wood (0.5 by 2 by 10 cm) placed in the center of each tray which provided an oviposition

Table 1. Flight response of mated female screwworm flies to bacteria-inoculated blood incubated for 24 to 96 h and uninoculated control expressed as number of females (mean ± SD) landing on treated and control samples and percentage females responding

Age, reproductive status, and ovarian stages	Incubation duration, h	No. females landing		*t*-values[a]	% responding[b]
		Treated	Control		
4-d-old, fertile, 80% in stage 7–8, 20% <stage 7	24	2.4 ± 2	1.5 ± 0.6	1.69	0.88
	48	4 ± 0.1	1.4 ± 1	2.49*	2.63
	72	3.6 ± 1.3	1.5 ± 0.6	2.32	2.13
	96	3.3 ± 1.3	1.1 ± 0.9	2.86*	2.13
7-d-old, fertile, 95% stage 10, 5% <stage 10	24	16.6 ± 1.4	1.4 ± 1	21.76*	15.25
	48	31 ± 2.7	2.3 ± 1.1	24.43*	28.75
	72	53.6 ± 5.3	2.1 ± 1.5	30.66*	51.5
	96	24.9 ± 3.9	2.5 ± 1	15.29*	22.38
10-d-old, fertile, all in stage 10	24	15.5 ± 3.8	2.4 ± 1	7.35*	13.13
	48	31.4 ± 4.3	2 ± 1	18.58*	29.38
	72	48.5 ± 5.7	87 ± 1	22.91*	46.63
	96	18.5 ± 2.6	1.37 ± 1	17.29*	17.13
7-d-old, sterile, 20% stage 3, 80% <stage 3	24	3.3 ± 1.2	3 ± 1.3	0.6	0.25
	48	16 ± 2.3	6 ± 2	12.33*	13.38
	72	18.9 ± 3.9	2 ± 1.3	13.54*	16.88
	96	4.3 ± 2.7	2.9 ± 1.9	1.21	1.38

[a] For paired *t*-test (df = 7), mean significantly different than the control values (*, $P < 0.05$).
[b] Percentage response was calculated as number responded to treated − number responded to control ÷ number of flies tested (800) × 100.

substrate. After 1 h, trays were removed from the cage, the number of egg batches in each tray was counted, and the weight of eggs per tray was recorded. Six tests were conducted for each incubation period.

Dissections. Ovarian development was scored by using the scheme of Adams and Reinecke (1979). According to this scheme, females with stage 2 ovaries possess previtellogenic oocytes in terminal follicles and stage 10 ovaries exhibit terminal follicles with mature eggs that are ready to be deposited. To confirm mating status, spermathecae of females were checked for sperm. Dissections were also made of the alimentary canal of some of the flies that landed and/or oviposited to record if they had fed on blood upon landing on the sample tray.

Statistics. Treatment effects for response bioassay in terms of age, incubation duration and reproductive condition were evaluated with analysis of variance (ANOVA) using randomized block design followed by the Tukey range test to separate means after a significant *F* value (Sokal and Rohlf 1981, NCSS 1995). Paired *t*-tests were performed to determine the significant differences between the treated and control values of flight response and oviposition.

Results

Behavior of Flies. Normally flies were inactive when no test sample was present in the cage. More than 90% of the flies rested on the ceiling of the cage and the upper one-third of all the walls. A few flies (<10%) rested on the bottom of the cage at the start of some tests. When the uninoculated (control) sample was introduced into the cage, a low flight activity was normally observed. However, most of the flies returned to resting position in 1–2 min. A few flies were seen flying around the sample tray without landing on it. Flies landing on the tray were counted to exhibit a positive response. When the control tray was removed and the tray with the inoculated sample was introduced in cages containing males and young (4 d old) fertile females, there was little flight activity which could be compared with that of the control test. However, when the test sample tray was introduced into the cage of 7- and 10-d-old fertile females, vigorous flight activity ensued and almost immediately the flies started landing on the sample tray. Other activities such as probing blood with proboscis, feeding, extending ovipositor, and, in many cases actual oviposition activity followed. In the presence of the test sample, 7-d-old sterile females were somewhat subdued when compared with fertile females of the same age group. There was less flight activity, probing, and feeding. Although we did not notice any ovipositor extension, these females continued probing the blood with their proboscis and crawling around the edge of the blood sample, behaviors most common in the gravid females offered inoculated blood.

Bioassay for Flight Response. Males of all ages tested were unresponsive (<1%) to the inoculated blood regardless of incubation duration. Female flies responded to inoculated blood in varying degrees depending on their age, sex, and reproductive status (fertile or sterile) and on the duration of blood incubation (Table 1). Response of 4-d-old fertile females to inoculated blood incubated for 24, 72, and 96 h was not significantly different when compared with the response to the corresponding control, but such females showed statistically significant response when tested with inoculated blood incubated for 48 h when compared with the corresponding control. Responses of 7- and 10-d-old fertile females to inoculated blood of all incubation periods were significantly higher than to corresponding controls. Response of 7-d-old sterile females to 48-and 72-h incubated bacteria-inoculated blood was significantly higher than that of the corresponding controls but not so for 24-and 96-h incubated blood (Table 1). Responses of 7- and 10-d-old females

Table 2. Comparisons of responses within age groups, incubation durations, and reproductive status

Variables	No. responded, mean ± SD[a]	*F* (*P*)	*df*
Age, d			
4	24 ± 4a	12.74*	2
7	252 ± 126b	(0.007)	
10	227 ± 120b		
Incubation duration, h			
24	64 ± 59a	4.51*	3
48	133 ± 115b	(0.024)	
72	182 ± 176b		
96	85 ± 82a		
Reproductive status and age			
4-d-old fertile	24 ± 4a		2
7-d-old fertile	252 ± 126b		
7-d-old sterile	86 ± 63a		

[a] Mean ± SD in each group followed by the same letter are not significantly different ($P = 0.05$, Tukey).

to inoculated blood were significantly higher than that of 4-d-old females (Table 2). Females of all ages were least responsive to the blood incubated for 24 h (Table 1). Responses to blood incubated for 48 and 72 h were significantly higher than those to 24 and 96 h (Table 2). A comparison of the response of fertile females with that of sterile females shows that the response of 7-d-old sterile females was not significantly different than that of the 4-d-old fertile females but significantly less than that of the 7-d-old fertile females (Table 2).

Oviposition Tests. Although, all the blood samples stimulated oviposition, there were significant differences in the amount of eggs laid between the inoculated and control samples, except for the 24 h incubation period (Table 3). The inoculated blood incubated for 48 and 72 h yielded significantly more eggs than did the blood incubated for 96 h. The number of egg batches roughly corresponded to the number of females laying those egg batches; however, sometimes it was difficult to determine the exact number of females oviposited because of the overlapping and contiguous nature of egg batches in a small area. Those females captured during or immediately after oviposition had blood in the alimentary canal at dissection. No visible difference was noted between the guts of flies fed on inoculated and control samples.

Table 3. Oviposition by 7-d-old female screwworms landing on the trays containing bacteria-inoculated or uninoculated control blood during 1-h observation

Incubation period, h	Treatment	No. egg batches, (mean ± SD)[a]	Wt. of eggs, g (mean ± SD)[a]
24	Treated	3.17 ± 1.2	0.04 ± 0.0
	Control	2 ± 0.6	0.03 ± 0.0
48	Treated	20.33 ± 2.9*	1.32 ± 0.2*
	Control	7.17 ± 1.9	0.15 ± 0.1
72	Treated	23.17 ± 3.5*	1.38 ± 0.2*
	Control	7.83 ± 2.3	0.08 ± 0.0
96	Treated	12.5 ± 2.7*	0.97 ± 0.2*
	Control	5.83 ± 1.9	0.1 ± 0.1

[a] *, mean significantly different from the control with paired *t*-test ($P < 0.05$).

Discussion

The bacteria-inoculated and incubated blood produced volatiles which attracted and/or stimulated locomotion by gravid flies and, to a lesser extent, by similarly aged sterile females without mature eggs. Our bioassay gave consistent results throughout. The response duration of 15 min allowed attraction of sufficient flies for statistical analysis of the results. Many more flies responded in a preliminary 30-min test, but it became difficult to count the number landing on the sample tray. Our preliminary observation showed consistent behavior of flies during the 0900–1300 hour test period, indicating no effect of control test on subsequent behavior of flies (unpublished data). Previous authors used olfactometers for screwworm response studies, which provided good results (DeVaney et al. 1971, Adams et al. 1979, Holt et al. 1979). Because of the complicated nature of the olfactometers used by these authors, we attempted to develop and test a simpler bioassay method which can be used in the future for rapid assaying of fractions from volatiles of bacteria-inoculated blood. The present method of bioassay is simple, fast, and provides reproducible results with minimum non-oriented locomotion due to other possible stimuli such as moisture from the hot water tray. Our results clearly indicate that the bovine blood inoculated and incubated with bacteria isolated from screwworm-infested animal wounds is capable of stimulating gravid screwworm flies to land on the sample tray. Previous authors reported similar results using some of the same bacterial species with or without bovine blood and demonstrated oriented attraction to bacteria produced odors over at least short distances (DeVaney et al. 1973, Eddy et al. 1975, Hammack et al. 1987). We have not tested the response to individual species of bacteria and, thus, the relative importance of each of the eight test species of bacterium in attracting screwworm has not been established. Previous studies indicate that the *Proteus* and *Providencia* species are most important for attracting screwworm flies (DeVaney et al. 1973, Eddy et al. 1975, Hammack et al. 1987).

When we checked the efficacy of the inoculated blood of various incubation periods, we observed that the blood incubated for 48 and 72 h was most effective in attracting the flies, probably because of a higher number of bacteria in these samples producing relatively more attractive volatile molecules. Attractiveness decreased when the blood was incubated for 96 h. This was probably due to high mortality of bacteria resulting from an increase of populations during a long incubation period, possibly causing depletion of nutrient in the medium. Accumulation of a toxic metabolite might also have caused death of the bacteria, resulting in reduced production of the attractive volatiles. Previous authors have reported a relationship between the ovarian development and the female's response to the volatiles (Hammack et al. 1987, 1989; Hammack 1990). Our results agree with these authors in that the response increased with the increase of ovarian development and the calendar age. However,

in our studies, the 7-d-old sterile females, having ovarian stage 3 or less, responded in significantly higher numbers to inoculated blood than to control blood, but to a lesser extent than did 7-d-old fertile females. These females were far from being gravid, but their calendar age was equal to that of fertile gravid females. It is possible that although these females did not have mature eggs in the ovary, their olfactory mechanisms, used for feeding- and oviposition-related orientation, were well developed, thus enabling them to respond significantly to inoculated blood. Our preliminary field studies with mixed-age sterile flies and 48 h incubated inoculated blood showed similar trends, where we caught large number of sterile females with ovarian stages 1–3 (unpublished data).

Results of the oviposition tests (Table 3) indicated that the inoculated and incubated blood stimulates oviposition when the flies are in contact with the blood. The fact that the flies laid eggs in uninoculated blood indicates that the oviposition stimulating factor is also present in the uninoculated blood. This was also observed in a few response bioassay tests where gravid females laid few eggs in control (uninoculated) samples. For each type of test, dissection of flies captured during or immediately after oviposition revealed blood ingestion. Feeding on blood by ovipositing females was reported by other authors (Holt et al. 1979, Hammack 1990), who confirmed the existence of a feeding attractant in the blood. It is possible that the volatiles from inoculated blood are responsible for attracting the gravid flies to the potential oviposition substrate. Once the flies come in contact with the substrate, they are probably stimulated to oviposit by a contact stimulus, or by feeding, or both. Holt et al. (1979) and Hammack (1991) observed feeding of flies accompanied oviposition and suggested that chemical stimuli, other than attractant, are important at close range for the stimulation of screwworm oviposition.

Our results suggest that the volatiles from bacteria inoculated and incubated blood are potent stimuli for gravid screwworm flies. If the active components from the volatiles can be isolated and identified, they may serve as attractants for sampling gravid females in the field, and for attracting females for oviposition in the mass rearing program.

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